NUCLEOTIDE-DEPENDENT PROCESSES IN THE THYLAKOID LUMEN OF PLANT CHLOROPLASTS

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Linköping, 4 April, 2008
“If you have no results, you haven’t worked”

Anonymous
ABSTRACT

Plants, algae and photosynthetic bacteria are able to harvest the sunlight and use its energy to transform water and carbon dioxide to carbohydrate molecules and oxygen, both important to sustain life on Earth. This process is called photosynthesis and is the route by which almost all energy enters the biosphere. As most simple things in life, the process of photosynthesis is easily explained but unfortunately not that easy to reproduce. If we could, we would be living in a much different world with almost unlimited energy. Light energy is harvested by chlorophyll molecules, bound to proteins in the chloroplast thylakoid membrane and drives the oxygen-evolving complex, to extract electrons from water. Electrons are then transferred to NADPH through photosystem II (PSII) to cytochrome b₆f and photosystem I, the major photosynthetic protein complexes. The cytochrome b₆f complex also transfers protons into the lumenal space of the thylakoid. These protons together with those from water oxidation create an electrochemical gradient across the thylakoid membrane, which fuels the ATP synthase to produce ATP. ATP, NADPH and carbon dioxide are used during the dark reactions to produce sugars in the chloroplast stroma. The thylakoid lumenal space where the water oxidation occurs has until recently been viewed as a proton sink with very few proteins. With the publication of the genome of *Arabidopsis thaliana* it seems to be a much more complex compartment housing a wide variety of biochemical processes.

ATP is a nucleotide and the major energy currency, but there are also other nucleotides such as AMP, ADP, GMP, GDP and GTP. Chloroplast metabolism has mostly been associated with ATP, but GTP has been shown to have a role in integration of light harvesting complexes into the thylakoid. In this work, we have demonstrated the occurrence of nucleotide-dependent processes in the lumenal space of spinach by bringing evidence first for nucleotide (ATP) transport across the thylakoid membrane, second for nucleotide inter-conversion (ATP to GTP) by a nucleoside diphosphate kinase, and third the discovery that the PsbO extrinsic subunit of PSII complex can bind and hydrolyse GTP to GDP. The active PSII complex functions as a dimer but following light-induced damage, it is monomerised allowing for repair of its reaction center D1 protein. PsbO is ubiquitous in all oxygenic photosynthetic organisms and together with other extrinsic proteins stabilises the oxygen-evolving complex. We have modelled the GTP-binding site in the PsbO structure and showed that the GTPase activity of spinach PsbO induces changes in the protein structure, dissociation from the complex and stimulates the degradation of the D1 protein, possibly by inducing memonisation of damaged PSII complexes.

As compared to spinach, *Arabidopsis* has two isoforms of PsbO, PsbO1 and PsbO2, expressed in...
a 4:1 ratio. A T-DNA insertion knockout mutant of PsbO1 showed a retarded growth rate, pale green leaves and a decrease in the oxygen evolution while a PsbO2 knockout mutant did not show any visual phenotype as compared to wild type. Unexpectedly, during growth under high light conditions the turnover rate of the D1 protein was impaired in the PsbO2 knockout, whereas it occurred faster in the PsbO1 knockout as compared to wild type. We concluded that the PsbO1 protein mainly functions in stabilizing the oxygen evolving complex, whereas the PsbO2 protein regulates the turnover of the D1 protein. The two PsbO proteins also differ in their GTPase-activity (PsbO2 >> PsbO1). Although their amino acid sequences are 90% identical, they differ in the GTP-binding region which could explain the difference in their GTPase activity. Based on these data, we propose that the GTPase activity of PsbO(2) leads to structural changes in interacting loops and plays a role in the initial steps of D1 turnover such as the PSII monomerisation step.

The nucleotide-dependent processes we discovered in the thylakoid lumen raise questions of transporters to facilitate these processes. As stated earlier, we provided biochemical evidence of an ATP thylakoid transporter, and most recently have identified a transporter that may be important for the export of lumenal phosphate back to the stroma. More transporters for GDP, metal ions and others solutes have still to be identified.
Växter, alger och fotosyntetiska bakterier lärde sig för 2.5-3 biljarder år sen att absorbera solljusets energi och använda det för att omvandla vatten och koldioxid till kolväten. Denna process kallas för fotosyntes och är den enskilt största energibindaren på jorden. Ljusenergin absorberas av klorofyll-molekyler bundna till proteiner lokaliserade i kloroplastens tylakoid-membran som driver själva vattenspjälkningen. Elektroner från vattnet vandrar till NADPH genom de stora fotosystem-komplexen; Fotosystem II, Cytochrome b₆f komplexet och Fotosystem I. Cytochrome b₆f komplexet har också som funktion att låta protoner vandra till lumen, som är lokalisat inne i tylakoiderna. Dessa protoner tillsammans med protonerna från vattnet skapar en protongradient över membranet, vilket fungerar som drivmedel för ATP-syntasens ATP produktion. ATP, NADPH och koldioxid används i mörker-reaktionen för att producera sockerarter med väldigt få proteiner. Med publikationen av backtravs genom har en mer komplex syn på detta utrymme vuxit fram.

Nukleotiden ATP, är den huvudsakliga energitransportören nukleotid, men det finns också andra nukleotider, till exempel AMP, ADP, GMP, GDP och GTP. Kloroplastens metabolism har för det mesta blivit kopplat till ATP, men GTP har visat sig ha en roll vid integrationen av ljusuppsamlande komplex in i tylakoid-membranet. I detta arbete har vi demonstrierat förekomsten av nukleotid-beroende processer i lumen; för det första genom nukleotid (ATP) transport över membranet; för det andra via nukleotid-konvertering, ATP till GTP, med hjälp av en Nukleosidediphosphatekinase i lumen; för det tredje, upptäckten att proteinet PsbO, som binder in till PSII och optimerar och skyddar vattenklyvningen, kan binda och hydrolysera GTP till GDP.

De fotosyntetiska organismerna, särskilt växter, har svårt att röra på sig och kan inte fysiskt skydda sig från överexponering av ljus och väder. Ljus, som är fotosyntesen drivkraft, är också en direkt fara. För starkt ljus ger skada på fotosystem-komplexen och för att skydda sig för det har flera skyddsåtgärder ”uppfunnits”. Reaktionscenter D1 proteinet är det protein som utstår mest skada och har en av cellens snabbaste omsättning. Det aktiva PSII komplexet, som fungerar som en dimer, mäste monomerisera innan det skadade D1 proteinet kan bytas ut. PsbO finns närvarande i alla fotosyntetiska organismer och stabiliserar vattenklyvningens komplexet. GTP aktiviteten av spenats PsbO ger strukturförändringar, dissoiering från PSII och stimulerar degradering av D1 proteinet. Arabidopsis thaliana har till skillnad från spenat, två isoformer av PsbO; PsbO1 och PsbO2.
En knockout-mutant av PsbO1 visar ett långsammare växtsätt, ljusgröna blad och en minskning av syrgas-produktionen. PsbO2 knockout mutanter uppvisar däremot inte några skillnader jämfört med vildtyps plantor. Oväntat så hade PsbO2 knockout mutanten, växande under starkt ljus nedsatt D1 reparation medan det skedde fortare i PsbO1 knockout mutanter när man jämförde det mot vildtyp. Vi kan dra slutsatsen att PsbO1 proteinets huvudsakliga funktion är att stabilisera syrgaskomplexet medan PsbO2 proteinets reglerar D1 proteinets reparations-cykel. Dessutom skiljer sig Arabidopsis isoformer av PsbO i deras GTPase aktivitet (PsbO2 >> PsbO1). Fast deras aminosyra-sekvens är 90% identisk, skiljer de sig i GTP bindande områden, vilket skulle kunna förklara aktiviteten. Baserat på detta föreslår vi att PsbO(2)s GTPase aktivitet leder till struktuella förändringar och spelar en viktig roll i monomeriseringsteget av PSIIs D1 protein reparations-cykel.

De nukleotidberörande processer vi visat ger upphov till fler frågor om transportörer över tylakoid-membranet, som kan transporter in och ut nödvändiga ämnen för processerna. Vi har visat förekomsten av en ATP thylakoid transportör och vi har nyligen identifierat en fosfattransportör som kan ha en viktig roll för regulationen av Pi innehållet i lumen. Andra transportörer för till exempel GDP, metalljoner och andra lösliga ämnen finns fortfarande kvar att upptäcka.
This thesis is based on the following publications, which will be referred to in the text by their Roman numerals (I-V).


*These authors equally contributed to this work.


OTHER PUBLICATIONS

### ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ATP</td>
<td>adenosine 5'-triphosphate (energy carrier produced during photosynthesis)</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine 5'-diphosphate</td>
</tr>
<tr>
<td>CP</td>
<td>chlorophyll-binding protein</td>
</tr>
<tr>
<td>CD</td>
<td>circular dichroism</td>
</tr>
<tr>
<td>Cyt b,f</td>
<td>cytochrome b,f</td>
</tr>
<tr>
<td>Chl</td>
<td>chlorophyll</td>
</tr>
<tr>
<td>D1</td>
<td>32 kDa D1 subunit of the photosystem II reaction center</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid (where genes are coded)</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine 5'-triphosphate</td>
</tr>
<tr>
<td>kDa</td>
<td>kilo Dalton (weight unit for proteins)</td>
</tr>
<tr>
<td>LC</td>
<td>liquid chromatography</td>
</tr>
<tr>
<td>LHC</td>
<td>light-harvesting complex</td>
</tr>
<tr>
<td>MALDI</td>
<td>matrix-assisted laser desorption ionization</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>m/z</td>
<td>mass over charge ratio (x-axis in MS spectrum)</td>
</tr>
<tr>
<td>NADPH</td>
<td>nicotinamide adenine dinucleotide phosphate, reduced form (reducing potential produced in photosynthesis)</td>
</tr>
<tr>
<td>NDPK</td>
<td>nucleoside diphosphate kinase</td>
</tr>
<tr>
<td>OEC</td>
<td>oxygen-evolving complex</td>
</tr>
<tr>
<td>Pi</td>
<td>inorganic phosphate</td>
</tr>
<tr>
<td>Psb(O,P,Q,R)</td>
<td>33, 23, 16, 10 kDa extrinsic subunits of the photosystem II complex</td>
</tr>
<tr>
<td>psbo(1,2)</td>
<td>knockout mutants of PsbO(1,2)</td>
</tr>
<tr>
<td>PSI</td>
<td>photosystem I</td>
</tr>
<tr>
<td>PSII</td>
<td>photosystem II</td>
</tr>
<tr>
<td>PQ</td>
<td>plastoquinone</td>
</tr>
<tr>
<td>RC</td>
<td>reaction center</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>TAAC</td>
<td>thylakoid ATP/ADP carrier</td>
</tr>
<tr>
<td>TOF</td>
<td>time of flight (detector in MS)</td>
</tr>
<tr>
<td>WT</td>
<td>wild type</td>
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CHAPTER 1

1. Introduction

1.1. Photosynthesis: an overview

Plants, algae and photosynthetic bacteria are able to harvest sunlight and use its energy to transform water and carbon dioxide to carbohydrate molecules and oxygen, both important to sustain life on Earth. This process is called photosynthesis, and has evolved 2.5 to 3 billion years ago from being dependent on substrates such as H$_2$S, NH$_3$ and Fe$^{2+}$, to oxidising H$_2$O (McFadden, 1999; Olson, 2006). It is the route by which almost all energy enters the biosphere. Sunlight energy is harvested by chlorophyll molecules bound to hydrophobic proteins, located in the thylakoid membrane and drives the oxygen-evolving complex, where the water-splitting reaction occurs (Lichtententhaler, 1987; Olson, 2006; Barber, 2007). Electrons are then transferred to NADPH through photosystem II (PSII) via the cytochrome b$_6$f complex (Cyt b$_6$f) and finally to photosystem I (PSI), the major photosynthetic protein complexes together with the ATP synthase. The Cyt b$_6$f complex also transfers protons into the lumenal space of the thylakoid, in conjugation with the electron transport. These protons together with those from water oxidation generate a proton gradient across the thylakoid membrane, which fuels the ATP synthase to produce ATP. ATP, NADPH and carbon dioxide are then used in the dark reactions to produce sugars in the chloroplast stroma. The thylakoid lumenal space where the water oxidation occurs has until recently been viewed as a proton sink with very few proteins. With the publication of the genome sequence of *Arabidopsis thaliana* (AGI, 2000) and proteomics works (Peltier et al., 2002; Kieselbach and Schröder, 2003) it seems to be a much more complex compartment housing a wide variety of biochemical processes.

1.1.1. Chloroplast

The chloroplast is the photosynthetic organelle in plants and green algae. It is surrounded by a double membrane (outer and inner envelope), and contains the soluble stroma and thylakoids (figure 1). The chloroplast, similarly to mitochondria, has its own genome, and thus the ability to synthesise essential proteins for the photosynthetic machinery. However, a significant number of chloroplast proteins are nuclear-encoded. For example, 10-15% of the nuclear gene products are predicted to be targeted to the chloroplast (Peltier et al., 2002). Proteins that are targeted to the
chloroplast have an N-terminal chloroplast transit peptide (cTP), which is processed when the precursor protein is translocated across the chloroplast inner envelope. Proteins then have two options; either remain in the stroma or move further into the thylakoid membrane or lumen. Proteins in the thylakoid lumen are targeted and transported via an additional transit peptide, the luminal transit peptide (lTP), which is located directly after the cTP. Soluble luminal proteins with lTPs are translocated by at least two different pathways: Sec pathway (ATP-dependent) and twin-arginine translocation (tat) pathway (pH-dependent) (Robinson et al., 2001; Peltier et al., 2002).

1.1.2. Thylakoid

The thylakoid membrane in plants can be divided into two different structures, namely grana and stroma lamellae (Menke, 1962; Menke, 1990). The grana regions usually cluster together into grana stacks, made up from repeating units that contain paired layers. These grana stacks can be further divided into surface-exposed, core and margin regions (Nelson and Yocum, 2006). The grana stacks are interconnected by the stroma lamellae (figure 1). Recently, the three dimensional structure of the plant thylakoid membrane has been revealed using electron tomography (Shimoni et al., 2005). In this structure the top layer bends slightly upwards and fuses with the layer above it and the other layer bends downwards and fuses with the layer below it. This proceeds with a counter clockwise rotation of 25° around the axis (figure 1). The thylakoid harbours the four photosynthetic complexes, PSII, Cyt b6f, PSI and ATP synthase driving the light reactions. These complexes are located differentially in the thylakoid membrane. The dimeric PSII complex is located in the grana, PSI and the ATP synthase are located in the stroma lamellae and the surface-exposed grana, whereas Cyt b6f is evenly distributed across the two thylakoid structures (Anderson, 1980; Allen and Forsberg, 2001; Anderson, 2002). However
other reports suggest that Cyt b₆f, which transports electrons between PSII and PSI, might mostly be localised in the grana margins (Allen and Forsberg, 2001).

1.1.3. Lumen

The thylakoid lumenal space is located inside the thylakoid and is continuous between the grana and stroma lamellae, as revealed in the recent 3-D structure (Shimoni et al., 2005). It has been predicted to contain around 200 proteins, although no more than 80 proteins have been identified using proteomics (Peltier et al., 2002; Schubert et al., 2002). There is also tremendous evidence for various biochemical activities in the lumen, such as chaperones (Schlicher and Soll, 1996), immunophilins (Fulgosi et al., 1998), carbonic anhydrases (Park et al., 1999), violaxanthin deepoxidases (Hieber et al., 2000), peroxidases (Kieselbach et al., 2000) and proteases (Adam, 2001). This growing complex view of the thylakoid lumen also raises the question of nucleotide-dependent processes in this chloroplast compartment.

1.2. Photosynthetic energy conversion

Photosynthesis is a unique and complex process converting sunlight into chemical energy. This process can be divided into two sets of reactions, the light and the dark reactions. In the light-driven reactions, light energy is used by chlorophyll bound to proteins located in the chloroplast thylakoid membrane, to drive the oxygen-evolving complex to form ATP from ADP, reduce electron carrier molecules, such as NADP⁺ to NADPH, and produce oxygen as a by-product. The dark reactions, called so because they proceed without the need for light, represent the second part of photosynthesis, where the energy of ATP and reducing power of NADPH are used for carbon dioxide fixation into sugars (Barber, 2007).

An overall equation of the oxygenic photosynthetic reactions is the following:

\[
\text{CO}_2 + \text{H}_2\text{O} + \text{light} \rightarrow (\text{CH}_2\text{O})_n + \text{O}_2
\]

The photosynthetic pigments such as chlorophylls, carotenoids and phycobilins are embedded in the thylakoid membrane in units called photosystems (PS) (Lichtententhaler, 1987). One photosystem can contain 250-750 pigment molecules (Dunahay et al., 1984; Neale and Priscu, 1995), and consist of two subcomplexes, an antenna protein complex (LHC) and a reaction centre (RC) complex, that capture the light at different wavelengths. There are several types of chlorophyll, among which chlorophyll \(a\) occurs in all photosynthetic eukaryotes and cyanobacteria. Chlorophyll \(b\) is found in vascular plants, bryophytes, green algae and euglenoids,
and functions as an accessory pigment to broaden the spectrum of the light that can be absorbed. When a chlorophyll \( b \) molecule absorbs the light, the energy is transferred to chlorophyll \( a \) which leads the energy to the oxygen-evolving complex (OEC) (Mathews and Holde, 1990; Raven et al., 1992).

When the light energy is absorbed by the pigments, the energy is transferred until it reaches the reaction center, which has one special pair of chlorophyll molecules. When one of these two chlorophylls absorbs the light energy, it loses one electron which is then transferred to a primary acceptor molecule, pheophytin (Pheo) (Raven et al., 1992).

There are two different photosystems in algae and plants, called photosystem II and I, having their own reaction center molecules, P680 and P700, with maximum absorption at 680 and 700 nm, respectively. In both photosystems, the primary step driven by the light energy is a charge separation from where one electron is provided to the electron transfer chain (figure 2) (Baker et al., 2007).

The water-splitting reaction takes place on the luminal side of PSII complex, and is performed by the OEC complex, composed of four Mn\(^{2+}\) and one Ca\(^{2+}\) ion (figure 2). Water is split into protons, oxygen and electrons. A tyrosine residue (Yz), positioned on the D1 protein, functions as an electron transfer intermediate between P680 and the OEC complex. It reduces by electron transfer P680 to its ground state, while itself is reduced by an electron from the splitting of water.

![Figure 2. A schematic representation of the electron and proton transfer chain and of the photosynthetic subunits involved.](image-url)
of water (Baker et al., 2007).

Electrons are transferred via redox components through PSII to the Cyt b₆f and PS I, where the second type of charge separation occurs, involving P700 (figure 2). Electrons are transferred to the primary acceptor Chl A₀, then to PhQ A₁, FeS₆, FeSₓ and FeSₓ₀ from where they leave PSI and reduce ferredoxin (Fd). The reduced ferredoxin reduces NADP⁺, via ferredoxin-NADP⁺ oxidoreductase to NADPH (Merchant and Sawaya, 2005). The Cytochrome b₆f complex also transfers protons into the luminal space of the thylakoid. The proton gradient across the thylakoid membrane drives the ATP synthase. ATP together with NADPH are used during carbon dioxide fixation (Mathews and Holde, 1990; Raven et al., 1992) (Baker et al., 2007). For details of the light reactions, see the recent review by (Merchant and Sawaya, 2005).

Photosynthetic organisms, especially plants, have a hard time to cope with the environment. They are literally rooted to the ground and cannot with ease move from their spot. Sunlight, which drives photosynthesis, can be damaging to the photosynthetic machinery and especially when the plants are exposed to high radiation. To overcome this problem, they have developed several protection systems; from turning of leaves to extensive repair systems and movement of the LHCII between PSII and PSI.

1.3. Photosynthetic complexes

The four photosynthetic complexes are the PSII complex, Cyt b₆f complex, the PSI complex and the ATP synthase. Among them, the PSII complex has been the main focus of research in the present investigation. There is overwhelming structural and functional information about each of these complexes, for recent reviews see (Dekker and Boekema, 2005; Nelson and Yocum, 2006).

1.3.1. Photosystem II

Photosystem II (PSII), a multi-subunit membrane–protein complex, uses light energy to perform the most thermodynamically demanding and unique reaction of photosynthesis, namely the oxidation of water to molecular oxygen and reducing equivalents. X-ray crystal structures obtained for the PSII complex isolated from *Synechococcus elongatus*, *Thermosynechococcus vulcanus* and *Thermosynechococcus elongatus*, under various resolutions (3.8 to 3.0) (Zouni et al., 2001; Kamiya and Shen, 2003; Ferreira et al., 2004; Loll et al., 2005) (figure 3). The PSII dimer has a 105 Å of depth, 205 Å of length and 110 Å of width at 3.5 Å resolution, and a molecular mass of 650 kDa (Ferreira et al., 2004). Each monomer contains more than 20 different proteins where 16 are intrinsic and four are extrinsic (Hankamer et al., 1997; De Las Rivas et al., 2004). Based on these,
a homologous refined structure of the LHCl-PSII supercomplex of higher plants was recently presented (figure 3) (Nield and Barber, 2006).

The assembly of PSII subunits of plants is regulated by the nucleus, but the core of the integral PSII subunits are encoded by the chloroplast genome. These include D1, D2, CP43 and CP47 proteins. For a review, see (Kessler and Schnell, 2006; Suorsa and Aro, 2007; Suorsa M, 2007).

The reaction centre of PSII consists mostly of two homologous proteins named D1 and D2, which bind cofactors for the electron transport. Closely associated to the D1 and D2 proteins are two chlorophyll-binding proteins called CP43 and CP47 (Barber, 2006). The OEC complex is located on the luminal side of the PSII complex (Seibert et al., 1987). In plants and green algae, there are four extrinsic proteins, PsbO, PsbP, PsbQ and PsbR associated with the OEC (Seidler, 1996; De Las Rivas et al., 2007). Cyanobacteria have PsbU and PsbV, as well as PsbO as OEC extrinsic proteins, although there is evidence that they may also have homologs of PsbP and PsbQ, associated with their PSII complexes (Thornton et al., 2004).

The water splitting in the RC of PSII has its side effects: the production of single oxygen and of highly oxidising redox components, which may cause damage to the PSII, and especially to its reaction centre D1 subunit. To survive, the D1 protein undergoes an extensive repair cycle, including degradation and replacement by a newly synthesised protein (Aro et al., 1993).

There are two mechanisms of D1 degradation, a donor and an acceptor-side inhibition. The acceptor-side (Aro et al., 1993) occurs under high light and is due to an over reduction of the
PQ pool. This in turn leads to the formation of P680 triplet state that can cause the formation of singlet oxygen. The single oxygen, produced in the vicinity of the D1 protein, leads to an excessive damage and the need for repair (Aro et al., 1993). The donor-side inhibition occurs when the donor-side of PSII can not keep up with the withdrawal of electrons from P680. Leading to long lived P680$^+$ and the oxidised tyrosine electron donor Y$_z^+$, which causes an inactivation of the PSII electron transport (Aro et al., 1993).

The D1 repair cycle starts with phosphorylation at its N-terminus by the STN8 kinase, activated by the reduction of the PQ pool; second, the oxidative damage to the D1 protein due to highly reactive singlet oxygen; third, the monomerisation of PSII and detachment of CP43, PsbP and PsbQ; fourth, migration of the monomerised PSII complex to the stroma lamellae; fifth, dephosphorylation of the D1 protein; sixth, multi-step degradation: first, by a primary cleavage by DegP2, resulting in a 23 kDa N-terminus and 10 kDa C-terminal fragments followed by a secondary cleavage by FtsH; seventh, a cotranslation insertion of a new D1 protein; eight, PSII monomer migration to grana and dimerisation of the PSII complex; ninth, activation of the PSII dimer to a functional state. (Adir et al., 1990; Aro et al., 1993; Spetea et al., 1999; Lindahl et al., 2000; Haußühl et al., 2001; Zaltsman et al., 2005; Kapri-Pardes et al., 2007).

1.3.1.1. Extrinsic proteins

The 33, 23, 16 and 10 kDa extrinsic proteins of PSII (PsbO, PsbP, PsbQ and PsbR) are associated on the luminal side of thylakoid membrane (figure 3). They were thought to be directly involved in oxygen evolution since the removal of the 23 and 16 kDa subunits led to a partial inactivation of the oxygen evolution (Seidler, 1996). The PsbO subunit, also called the manganese stabilising protein, is conserved in all known oxygenic photosynthetic organisms, showing a 40–50% sequence homology between cyanobacteria and higher plants. PsbO optimizes the ionic environment, provides a polar channel for protons/water, and stabilises the metal cluster (Seidler, 1996; De Las Rivas and Barber, 2004; Murray and Barber, 2007).

PsbP together with PsbQ have been thought to be responsible of Ca$^+$ and Cl$^-$ binding (Seidler, 1996), but a recent paper states that PsbP is required to maintain the active Mn$^{2+}$, Ca$^+$, Cl$^-$ cluster in vivo, and that PsbQ is not necessary for either PSII function or growth (Ifuku et al., 2005).

In the present investigation, the focus has been on the PsbO protein. A review by (De Las Rivas et al., 2007) goes through the other extrinsic proteins in more detail.
1.3.2. Cytochrome b₆f (Cyt b₆f)

The oxidation of plastoquinol occurs by an integral membrane protein complex, Cyt b₆f. It functions as a plastoquinone-plastocyanin oxidoreductase, transferring electrons from plastoquinol to plastocyanin, a copper-containing protein. This electron transfer is accompanied by the translocation of protons across the membrane, this creates a proton gradient across the thylakoid membrane, which drives the ATP synthesis in the stroma (Cramer et al., 2006).

1.3.3. Photosystem I (PSI)

The plant photosystem I (PSI) complex, is a monomer as compared to cyanobacterial PSI which is a trimer as to PSII which is a dimer, reviewed in (Melkozernov et al., 2006). PSI accepts electrons from the cyt b₆f complex via plastocyanin. PSI reaction centre Chl absorbs light with a maximum wavelength of 700 nm (P700). The electrons are transported through a chain of redox cofactors to the ferredoxin-NADP⁺ complex, completing the electron flow from H₂O to NADP⁺. PSI has a peripheral LHC (LHCI), but can share the LHCII with PSII. Under changing light conditions (state transitions) LHCII detaches itself from PSII and connects to PSI (Allen and Forsberg, 2001)

1.3.4. ATP synthase

There are three types of ATPases, P, F and V–type, where the F-type is located in the inner membrane of mitochondria and in the thylakoid membrane of chloroplasts. The sequence of thylakoid ATP synthase is termed CF₁CF₀ complex. By analogy with mitochondrial ATPase which transfers protons into the matrix, the thylakoid ATP synthase is driven by the H⁺ emigration from the luminal space to the stroma (Mathews and Holde, 1990). The mechanism of the proton-driven ATP synthesis can be described in three steps; first, translocation of protons carried out by F₀; second, catalysis of formation of the phosphoanhydride bond of ATP carried out by F₁; third, coupling of the dissipation of the proton gradient with ATP synthesis (Mathews and Holde, 1990).
CHAPTER 2

2. Functional genomics in plants

Identification and functional characterisation of proteins from organisms with sequenced genomes (e.g., Arabidopsis, (AGI, 2000)) involves several steps in a functional genomic approach. The prediction provides information about the sequence of the protein of interest, in which species it can be found, the putative function and where it can be localised. Also, by using bioinformatics, protein candidates for a certain biochemical activity can be found. Then, localisation- and characterisation studies in vivo and in vitro provide the experimental validation of the predictions. An important part of the characterisation is represented by phenotypic studies of knockout mutants for the gene candidate.

2.1. Prediction studies

There are numerous databases, servers and tools that can be accessed over the Internet or used as a stand alone program; a few of them and routine analyses are listed below.

2.1.1. Databases and tools

Below, I briefly describe the databases and tools that I have used in the present investigation.

NCBI (https://www.ncbi.nlm.nih.gov/) was established in 1988 as a National Resource for Molecular Biology Information, creates public databases, conducts research in computational biology, develops software tools for analysing genome data, and disseminates biomedical information (NCBI).

ExPASy (http://www.expasy.ch/) is a server dedicated for the analysis of protein sequences and structures. Swiss-Prot is the most popular and used database available at this server (Gasteiger et al., 2003).

BLAST is a widely used tool at several servers (NCBI, ExPASy), and searches for homologies to sequences in the same organism and other species.

ClustalW is a tool found at EBI, used to align sequences and to identify conserved regions.
TargetP (http://www.cbs.dtu.dk/services/TargetP/) predicts the sub-cellular location of eukaryotic proteins. The location assignment is based on the predicted presence of any of the N-terminal presequences: chloroplast transit peptide (cTP), mitochondrial targeting peptide (mTP) or secretory pathway signal peptide (SP) (Nielsen et al., 1997; Olof Emanuelsson, 2000).

PyMOL (http://pymol.sourceforge.net/) is a PDB viewer program, based upon an opensource project, and visualises protein crystal structures saved in a pdf format (PyMOL, 2006).

CAVER (http://loschmidt.chemi.muni.cz/caver/index.php) calculates tunnels leading from cavities inside static or dynamic protein or protein complexes. It does this by calculating the least costing path, by giving numerical numbers corresponding to the resistance of closely located molecules. It can be used both as a plugin for the PyMOL program or as an online application (Petřek et al., 2006; Damborský et al., 2007).

Signal (http://signal.salk.edu/) is used to search for availability of public collection of Arabidopsis knockout mutants and the insert location (Alonso et al., 2003).

Geneinvestigator (https://www.genevestigator.ethz.ch/) is a reference expression database and meta-analysis system. It allows to study the expression and regulation of genes in a broad variety of contexts by summarizing information from hundreds of microarray experiments into easily interpretable results (Zimmermann et al., 2004; Zimmermann et al., 2005).

### 2.2. Photosynthetic preparations

For the main parts of this thesis, *Arabidopsis thaliana* has been used as plant material (papers I, III and IV). Spinach was used in the earlier stages of this work (papers I and II).

*Arabidopsis* is a small flowering plant that was discovered by Johannes Thal already in the sixteenth century. It is favoured by scientists for its small genome which was fully sequenced in 2000. The genome has a total of 125 Mbp with 25,498 genes, encoding proteins belonging to 11,000 families (AGI, 2000).

Isolation of chloroplasts, thylakoids and various PSII components are described in the attached papers. Here I provide a summarised flow scheme (figure 4); first, the leaves are homogenised in a mixer; second, chloroplasts are isolated through centrifugation and washing steps; third, thylakoids are extracted by the disruption of the chloroplast envelope using osmotic shock followed by differential centrifugation; forth, using thylakoids, various preparations
can then be obtained: LHCII-PSII supercomplexes, PSII membranes (BBY), PSII cores and the soluble lumen content, by using detergents or mechanical pressure combined with various centrifugation steps. There are major differences between the different types of preparations. The PSII membranes are fragments from the grana regions enriched in LHCII-PSII dimeric complexes, and containing all of the OECs extrinsic proteins. PSII cores are PSII monomers largely devoid of LHCII as well of all extrinsic proteins except for PsbO.

2.3. Localisation studies
To localise your protein of interest, there are many standard techniques; those used in the present investigation are listed below.

2.3.1. Sodium dodecyl sulfate polyacrylamide gel electrophoresis, SDS-PAGE
Gel electrophoresis is a technique where proteins are separated in a solid matrix using an electric field. The rate of the migration is based on the protein net charge, size, electric field, and also on
the composition of the mediums (Westermeier, 2001). The most common used variant is SDS-PAGE, a discontinuous system originally described by Laemmli (Laemmli, 1970). SDS is an anionic detergent, which binds strongly to and denatures proteins. SDS removes the intrinsic charge of the protein, and a constant negative net charge per unit is obtained. When run under the same conditions on a polyacrylamide gel, different sized proteins will travel at various speed, and therefore be separated on the gel.

2.3.2 Immuno detection
The protein of interest can be probed with antibodies raised against a homologous protein from another organism or against a specific peptide from this protein. The protein, that can be in an isolated form or associated to a complex, can be detected by Western blotting or fished out using immunoprecipitation.

2.3.3. Radioactive labelling
Radioactive assays are used to give experimental proof of protein function. The methods use radioactively labelled compounds such as $^{14}$C, $^{32}$P, relevant for the function of the protein. The radio-labelled protein is detected on a phosphorimage of SDS-gels. The radio-labelled nucleotides are detected by phosphorimage of a thin layer chromatographic plate. The amount of radioactivity is determined by liquid scintillation spectrometry.

2.3.4. Organelle import
Organelle import is a classic method for localisation studies. The protein of interest is synthesized in vitro in a radiolabelled precursor form, containing a targeting peptide at its N-terminus that directs the protein for example into the chloroplast. The protein can also be synthesised as a fusion construct with a recognition label at the C-terminus, such as a green fluorescent protein (GFP) tag, which can be visualised in a fluorescence microscope.

2.3.5. Mass spectrometry
The protein of interest can be enzymatically cleaved and extracted from a protein mixture, SDS-gel or membrane and the obtained peptides can be analysed without breaking the covalent structure of these molecules by mass spectrometry (MS) techniques. In the matrix-assisted laser desorption ionization (MALDI) technique (Tanaka et al., 1988), and further developed in the time-of-flight (TOF), ionized peptides are accelerated in an electric field and enter a flight tube. During the flight in this tube, different molecules are separated according to their mass to charge ratio.
ratio (m/z) and reach the detector at different times. The masses of the peptides are then analysed and searched in various databases. MS techniques can also be used for quantitative applications, for example to compare the levels of a certain protein in knockouts mutants and wild type plants (Edvardsson et al., 2007).

2.4. Structural analyses

There are four different aspects of the structure of a protein. Its primary structure is the amino acid sequence of the polypeptide chain. The secondary structure (sub structure) shows the \( \alpha \)-helix, \( \beta \)-sheet and random coils distribution in the protein. The tertiary structure shows how the protein is folded in space. The quaternary structure shows how proteins are folded together in complexes. Different techniques listed below have been used to investigate protein structure in this thesis (Mathews and Holde, 1990).

2.4.1 Circular dichroism

Circular dichroism (CD) is a technique to characterise the secondary structure of proteins. The CD spectra are generated based on the optical spectra of the polypeptide chain. When a protein is exposed to circularly polarised light the different structures of the protein give characteristic bands, reflecting the electronic excitation energies. Secondary structure elements such as the \( \alpha \)-helix, \( \beta \)-sheet and random coil all give specific bands. This analysis can therefore lead to the determination of the secondary structure of a protein. CD can also be used to detect conformational changes induced with substrates (Bulheller et al., 2007).

2.4.2. Intrinsic fluorescence

Intrinsic fluorescence of the aromatic amino acids tryptophan, tyrosine and phenylalanine is used to monitor structural changes in proteins. Among them, tryptophan has the highest fluorescence and quantum yield. The tryptophan fluorescence is used as a tool to determine if the residue is buried in the protein core or exposed on the surface, and thus to investigate changes in the tertiary structure of a protein during folding and substrate binding. For reviews, see (Cioni and Strambini, 2002; Bulheller et al., 2007).
2.5. Phenotypic analyses

2.5.1. Plant fitness
The overall fitness of knockout mutants has been compared to that of wild type (WT) plants during growth under normal (120 µmol photons m\(^{-2}\) s\(^{-1}\)) and under high light (1000 µmol photons m\(^{-2}\) s\(^{-1}\)).

2.5.2. Leaf weight
The weight of leaves is in direct correlation with how large they are. Comparing the leaf weight in plants under various growth conditions, as in paper III, may provide indications for an impaired function. The reduced growth of a knockout mutant points to impaired functions due to the absence of an important protein.

2.5.3. Chlorophyll \(a/b\) ratio
To determine total chlorophyll Chl (\(a+b\)) concentration and Chl \(a/b\) ratio of a photosynthetic preparation, samples are measured in a spectrophotometer at specific wavelengths, 663 and 645 nm, corresponding to maximum absorption by chlorophyll \(a\) and chlorophyll \(b\), respectively. The equation of Amon (Amon, 1949) was previously routinely used, but recently the equation of Porra (based upon a modification of Amon) has gained popularity. A ratio of around 3 is typical for thylakoids, around 2 for isolated PSII membranes and greater-than or equal to 4 for PSII cores (Porra, 2002).

2.5.4. Chlorophyll content
The chlorophyll content of a photosynthetic tissue gives indications about the rate of senescence as well as the efficiency of photosynthesis. For comparing young to mature and senescent tissues, a ratio of the chlorophyll content per mass of tissue is calculated.

2.5.5. Oxygen evolution
Molecular oxygen produced during water oxidation by OEC of PSII, can be used as an indicator how well the photosynthetic apparatus performs. It can be measured by using an oxygraph with a Clark-type electrode. By using saturated light and electron acceptors, which withdraw electrons from different parts of the photosynthetic machinery, we can locate the irregularity in the electron transfer chain.
2.5.6. Chlorophyll fluorescence

The light energy that is absorbed by the leaf may take three directions; it can be dissipated as heat, drive photosynthesis or can be emitted as fluorescent light. The emission of fluorescent light from the leaf is called chlorophyll fluorescence. Although the emitted light is low (only 1-2% of the total light absorbed), it can be measured using a fluorometer. The three directions are always in conjugation with each other, if one goes up the others go down so that the energy always is the same as the light energy coming in. By measuring the yield of chlorophyll fluorescence, information about the other directions can be gained, as reviewed in (Maxwell and Johnson, 2000).

Once PSII has absorbed light and Q$_A$ has accepted one electron, it is not able to accept another one until it has passed the first electron to the next electron carrier Q$_B$. During this time, the reaction centre is called “closed”. In darkness the reaction centers are open and by exposing them to light they will gradually be closed. An increase in the ratio between closed and open reaction centers leads to an increase in the yield of chlorophyll fluorescence.
CHAPTER 3

3. Present Investigation

3.1. Aim of this study

The lumenal space, of the thylakoid was known in the past to harbour just a few proteins, but recent biochemical and proteomic reports point to a much more active role for this compartment in photosynthetic regulation. This new and complex view of the lumenal space raises several questions of the chloroplast function and regulation. One of these questions concerns the presence of nucleotides, nucleotide-binding proteins and nucleotide regulation in the thylakoid lumen of plant chloroplasts, which was a general aim of this work.

This chapter summarises the findings of the papers I to V. A short description of the aim of each paper is listed below.

Paper I: To identify nucleotide-dependent reactions in the thylakoid lumen of spinach chloroplasts and an ATP transporter in thylakoid membrane.

Paper II: To characterise the GTP-binding properties and GTPase activity of spinach PsbO.

Paper III: To study the roles of Arabidopsis PsbO isoforms.

Paper IV: To investigate if Arabidopsis PsbO isoforms differ in their GTPase activity.

Paper V: To identify and functionally characterise a phosphate transporter in the Arabidopsis thylakoid membrane.
3.2. Working model

![Working model during the present investigation](image)

**Figure 5. Working model during the present investigation**

1. ATP import and ADP export across the thylakoid membrane, via the thylakoid ATP/ADP carrier, TAAC (Paper I)
2. Intra-conversion of ATP to GTP, by transfer of the $\gamma$-phosphate from ATP to GDP via Nucleoside diphosphate kinase, NDPK3 (Paper I)
3. GTP binding and hydrolysis by the PSII extrinsic PsbO protein (Papers I, II and IV)
4. Following light-induced damage to PSII, the PsbO1 protein of *Arabidopsis thaliana* is exchanged for the PsbO2 protein. The GTPase activity of the PsbO2 protein (PsbO in spinach), initiates the monomerisation of the PSII dimer as an initial step of D1 turnover (Papers III and IV)
5. Lumenal Pi is exported to the stroma via the thylakoid anion transporter 1, ANTR1 (Paper V)
3.3. Nucleotide-dependent processes in the chloroplast

The outer and inner envelope of the chloroplast host several nucleotide-dependent processes and have the function to transport and exchange proteins, solutes and metabolites between the cytosol and the stroma. Most chlorophyll proteins are nuclear-encoded and need to be imported through these membranes from the cytosol. Two protein complexes are responsible for the protein import, namely the translocon at the outer envelope membrane of chloroplasts, Toc, and the translocon at the inner envelope membrane of chloroplasts, the Tic apparatus. Toc binds the preprotein, coming from the cytosol, in the presence of ATP and GTP and the preproteins transit peptide associates with the Tic complex and forms a Toc and Tic supercomplex, reviewed in (Chen and Schnell, 1999; Spetea and Thuswaldner, 2008). The insertion of the protein from the Tic complex is made by the help of hydrolysis of stromal ATP. In addition to protein import, the inner envelope holds other nucleotide-dependent processes such as lipid translocation and transporters (Lu et al., 2007; Spetea and Thuswaldner, 2008).

Inside the chloroplast are nucleotides, such as ATP, produced by the ATP synthase on the stromal side of the thylakoid membrane. These are involved not just in the photosynthetic dark reactions but also play an important role in energy-dependent processes such as phosphorylation, transport, degradation and folding of proteins (Spetea and Thuswaldner, 2008). Protein phosphorylation on the stromal side of the thylakoid membrane has an important role in how photosynthetic organisms cope with the environment. Excess light to PSI relative to PSII leads to the reduction of the PQ pool (Vener et al., 1997) and the phosphorylation of the light harvesting complex signals for a reversible association of LHClI between PSII and PSI, called state transition (Zito et al., 1999; Bellaﬁore et al., 2005). During high light stress, PSII core proteins, especially the PSII D1 protein that harbours most of the redox components get phosphorylated, dephosphorylated and degraded (Aro et al., 1993). The turnover rate of the D1 protein is the fastest known in chloroplast, with a half life of 30 minutes. The degradation step following light-induced damage has been shown to be nucleotide dependent (Spetea et al., 1999; Spetea et al., 2000).

While the stromal side of the thylakoid membrane has several nucleotide-dependent processes involved in the regulation of photosynthetic light reactions, there have been no reports on the presence of nucleotides in the thylakoid lumen before the initiation of the present investigation.

It has earlier been reported that the presence of GTP bound to the thylakoid membrane enhances the D1 degradation rate (Spetea et al., 1999; Spetea et al., 2000) In paper I, we have
attempted to find GTP-binding proteins in the thylakoid membrane with a role in D1 protein degradation (figure 5).

For this purpose, we incubated dark-controlled and pre-illuminated thylakoid membranes with $[^{32}\text{P}]8$-N$_3$GTP, which upon UV exposure binds covalently to polypeptides containing GTP-binding sites. Two radioactive bands of 33 and 36.5 kDa could be detected. The band of 33 kDa was detected mainly in the preilluminated thylakoids and to a lower extent in the dark sample. When isolated PSII cores were incubated with $[^{32}\text{P}]8$-N$_3$GTP, the 33 kDa protein was detected, but not the 36.5 kDa protein. The labelling of the 33 kDa band was inhibited by DCMU, which points to an involvement in the electron transport. The photolabelled 33 kDa was washed away by alkaline TRIS and crossreacted with an antibody against the OEC33 (PsbO) protein. Upon incubation of thylakoid membranes with $[^{32}\text{P}]8$-N$_3$ATP, only the 36.5 kDa was detected and stronger than compared with $[^{32}\text{P}]8$-N$_3$GTP, pointing to a higher affinity for ATP than GTP. Thylakoid subfractionation showed that the 36.5 kDa was mainly located to the stroma-exposed membranes.

Nucleoside diphosphate kinases (NDPK) catalyses the transfer of the $\gamma$-phosphate of a triphosphate nucleoside to a diphosphate nucleoside by a ping-pong mechanism. In plants several isoforms have been purified from cytosol (NDPK1) and chloroplast (NDPK2, 3) and mitochondria (mtNDPK) (Spetea and Thuswaldner, 2008). To test for NDPK activity, thylakoid lumen from spinach was incubated with $[^{32}\text{P}]$ATP in the presence and absence of externally added GDP. Radioactive GTP was detected only in the presence of GDP, and when incubated with $[^{32}\text{P}]$GTP radioactive ATP was detected. The $K_m$ for ADP was four-fold higher than for GDP, indicating a preference of lumenal NDPK for GDP. Bioinformatic searches indicated NDPK3 as a candidate for lumenal NDPK. Western blotting and immunoprecipitation with a peptide-specific antibody as well as chloroplast import studies validated the lumenal location of NDPK3.

Nucleotide metabolism and GTP binding to PsbO pointed to existence of nucleotide transport across the thylakoid membrane (figure 5, steps 1-3). To test if externally added ATP could be transported across the thylakoid membrane from the stroma to the lumen, intact thylakoid membranes were incubated with $[^{32}\text{P}]$ATP in darkness and under illumination. The thylakoids were washed and reisolated and tested for the presence of $[^{32}\text{P}]$GTP. As shown in paper I, radioactive GTP could be formed by the help of the lumenal NDPK3. When spinach thylakoids were immunoblotted with an antibody against the ADP/ATP carrier (AAC) protein from bovine mitochondria a major band of 36.5 kDa was detected. As detected earlier a corresponding band of 36.5 kDa was detected to bind ATP using $[^{32}\text{P}]8$-N$_3$GTP and $[^{32}\text{P}]8$-
N_{3}ATP. To verify that the 36.5 kDa nucleotide-binding protein was responsible for the transport, thylakoids were immunoprecipitated with the AAC antibody or preimmune serum. The AAC antibody reduced the NDPK mediated formation of GTP to 20% as compared with the preimmune serum 72%. This gives support to the fact that the 36.5 kDa band correspond to the nucleotide transporter homologous to the bovine AAC.

3.4. Plant PsbO as a GTPase

3.4.1. GTP-binding proteins

Small GTP-binding proteins (GTPases) have a molecular mass of 20-40 kDa and constitute a superfamily with more than 100 members. Small GTPases can be classified in five families: Ras, Rho, Rab, Sar1/Arf and Ran (Takai et al., 2001). The Ras GTPases are key regulators for several events such as differentiation, vesicle transport, nuclear assembly and signalling pathways, as reviewed in (Lunquist, 2006; Spetea and Thuswaldner, 2008). GTPase change from an active to an inactive state by binding a GTP molecule followed by hydrolysis to GDP (figure 6). The rate-limiting step of the GDP/GTP exchange reaction is the dissociation of GDP from the GDP bound form (Takai et al., 2001). Proteins regulating this event are known as GTPase activating proteins (GAP) which promote the inactive state and GTP exchange factors (GEFs) which facilitate the exchange of GDP for GTP, and thus favours the active state (Yang, 2002; Siderovski and Willard, 2005; Spetea and Thuswaldner, 2008). The GDP dissociation inhibitors (GDIs) reviewed in (DerMardirossian and Bokoch, 2005; Siderovski and Willard, 2005) are also regulators of GTPases. They have three functional activities; first, they inhibit the dissociation of GDP, thus holding the GTPase in its inactive form; second, GDIs can inhibit GTP hydrolysis; third, they can maintain GTPases in a soluble phase and thus preventing it to bind to the membrane.

Sequence analyses of small GTPases have revealed that GDP/GTP-binding proteins have well-conserved binding motifs. The phosphate binding loop (P-loop or G1) consists of a glycine rich sequence followed by a lysine and serine or threonine. (GX,GKS/T, PROSITE PS00017). This P-loop may interact directly with one of the \( \alpha/\beta \)-phosphates of the bound GTP/GDP (Saraste et al., 1990; Rensland et al., 1995). The binding regions called G2 and G3
interact with Mg\(^{2+}\) and γ-phosphate, respectively. G2 consists of an aspartic acid followed by two amino acids and one glycine (DX\(_2\)G), whereas G3 consists of a conserved Thr onine (Bourne et al., 1991; Leipe et al., 2002). A fourth conserved region (G4), involved in the binding of the guanine ring, consists of hydrophobic and polar amino acids followed by (N/T)KX(D/E) (Bourne et al., 1991; Leipe et al., 2002).

In paper I, we discovered that the 33 kDa subunit (PsbO) of the PSII complex in spinach could bind GTP. We further characterised spinach and Arabidopsis PsbOs as GTPases in paper II and IV.

3.4.2. GTP-binding regions of PsbO

PsbO is exposed to the lumenal side of the PSII where it is responsible for the stability of the Mn cluster of the OEC complex. Plant PsbOs structural information, have been gathered through various biophysical approaches (Xu et al., 1994; Svensson et al., 1996; Shutova et al., 1997). Cyanobacterial PsbO has two regions, a head domain that interacts with PSII and a β-barrel core (Ferreira et al., 2004). In figure 7, the PsbO protein structure of the homology spinach PsbO and Thermosynechococcus elongatus is shown, where the colours (red, blue, magenta) correspond to the GTP-binding motifs of GTP in spinach; G2-G3, G1 and G4 respectively. In the structure homology model of spinach PsbO based upon the X-ray crystal structure of cyanobacterial PSII (Ferreira et al., 2004), we have predicted two loops, as the Switch I and II, corresponding to the regions flanking G2 and G3 motifs, namely the long \(β_1-β_2\) and the short \(β_2-β_3\) (paper II). Switch I, interacts with CP47 on the luminal surface of the other PSII monomer constituting the dimeric complex (De Las Rivas J, 2004). A function for Switch II has so far not been reported, however it can be speculated to interact with another luminal protein. In figure 4, of paper II, we show a proposed location/orientation of the GTP molecule in the PsbO structure, namely inside the PsbO β-barrel domain. The surface of PsbO is rich in basic and acid residues, thus making it a hydrophilic protein, while the central part of the β-barrel is not hollow but full of bulky hydrophobic residues (De Las Rivas J, 2004). To search for a potential pocket inside the PsbO protein, we have used the CAVER program for finding a tunnel originating from the G2 domain. This program has been recently used to identify water and proton channels leading from the Mn cluster (Murray and Barber, 2007).

In this work we have found four potential tunnels in the homolous PsbO structure from spinach and five potential tunnels in the structure from Thermosynechococcus elongatus. Only one of them exited on the luminal side of both of the PsbO proteins (figure 7), between the loops of \(β3-β4\), \(β4-β5\) and \(β6-β7\) and inside the loop of \(β4-β5\), respectively. The average radius of
these tunnels was 2 Å and the luminal exiting tunnel had the least cost of the five. The luminal tunnel structure/cavity matches with prediction of the GTP-binding site in the PsbO structure. The tunnel inside the spinach PsbO has a different exit and is much less restricted than to the corresponding tunnel of *Thermosynechococcus*. The N-terminus tail of PsbO could function as a lid and control the binding or dissociation of GTP/GDP. Beside the location of the GTP-binding site inside the β-barrel, close to the luminal exit of the protein, it is important to emphasise that conformational changes in the Switch I (and II) loops upon GDP/GTP exchange may weaken the interaction between PSII monomers (figure 5).

3.4.3. Mg GTP induces structural changes in PsbO

To study the interaction of GTP and PsbO, the secondary structure was investigated by far-UV CD spectroscopy upon the addition of GTP to the spinach protein in solution. Figure 2 in paper II shows that GTP induces changes in the PsbO structure in the presence of Mg2+ while without the metal ion no structural changes are observed. Eukaryotic PsbOs contain a single tryptophan
residue Trp-241 in the mature form of spinach PsbO (figure 8), which intrinsic fluorescence is used to monitor structural changes in its surroundings. The differential spectra with or without MgGTP showed that the tryptophan fluorescence was affected and shifted to a shorter wavelength, indicating that this amino acid becomes more buried inside the core of the protein.

3.4.4. GTPase activity of PsbO in its isolated and associated form
GTP hydrolysis experiments of the PsbO protein were conducted in its isolated form or associated to PSII in different PSII preparations such as PSII membranes, NaCl-washed PSII membranes and PSII cores. The GTPase activity was measured in darkness at pH 6.0 and 7.4, corresponding respectively to the physiological pH under in vivo light and dark conditions in the lumen. Only PSII membranes and isolated PsbO proteins showed significant difference between the different pH conditions. By calculating the amount of PsbO per mol PsbO in the various PSII preparations we could give a better comparison of the GTPase activity, see figure 3C in paper II.

The results show that the PsbO protein has a low intrinsic GTPase activity, which is enhanced when associated to a PSII dimer. This point to the fact that the PsbO as a GTPase requires a certain conformational change, which is best achieved when bound to the dimeric form of PSII. The implication of this finding is a potential role of this activity in the PSII monomerisation step of D1 turnover (figure 5).

3.4.5. GTP stimulates the light-induced release of the PsbO protein and D1 degradation
Previously, it was reported that GTP enhanced the degradation of the D1 protein in photo-inhibited thylakoid membranes as well as PSII complexes (Spetea et al., 1999; Spetea et al., 2000). In paper II we show that GTP stimulates the light-induced release of PsbO protein under similar experimental conditions as those inducing D1 degradation.

Following GTP hydrolysis, the inactive GDP form of the protein may be released from the membrane. It has previously been known that the PsbO protein dissociates from the PSII complex upon light exposure (Hundal et al., 1990). In paper II, we have investigated potential roles of GTP in the mechanism of PsbOs dissociation from the PSII complex (figure 5, step 3-5). In figure 4A of paper II we show the effect of GTP on PsbO in dark and light-exposed NaCl-washed PSII membranes at pH 6.0 and 7.4. At pH 6.0 the PsbO release was stimulated by GTP in both dark and light exposed membranes, while at pH 7.4 the release was only stimulated in light-exposed membranes.
Figure 8. Prediction and location of GTP-binding domains in the structure of the PsbO protein, as shown in paper II. PsbO sequences from the following organisms were aligned using ClustalW software: Spinacia oleracea (spiol), Arabidopsis thaliana (Arath), Chlamydomonas reinhardtii (Chlam) and from Thermosynechococcus elongatus (Synel). Black boxes mark the changes in the amino sequence between PsbO1 and PsbO2 in Arabidopsis thaliana. The blue background marks the G1 domain, red the G2-G3 domains, magenta the G4 domain. The yellow background flanking G2-G3 domain indicates Switch I (Sw I) and Switch II (Sw II) regions. The eukaryotic tryptophan residue, located in the C-terminus of PsbO is indicated in green.

Figure 4B from paper II shows a decrease in oxygen evolution, upon light exposure and addition of GTP to LHCII-PSII supercomplexes, which have PSII in its dimeric form that retains all OEC proteins and have a high level of GTPase activity. The effect occurs upon exposure to various light conditions, from darkness, low, growth to highlight. Western blot of the supernatant and pellet of the membranes from the LHCII-PSII supercomplexes, showed an enhanced release of PsbO and corresponding degradation of the D1 protein at enhancing light intensities. The most remarkable effect of GTP was on degradation of D1 protein at 200 µmol photons m⁻² s⁻¹, corresponding to the largest effect of GTP on the release of PsbO.
3.4.6. Different roles of PsbOs in *Arabidopsis thaliana*

Paper I and II provided strong evidence for new roles for PsbO in addition to stabilising the OEC. As stated in chapter 1.3.1.1., *Arabidopsis thaliana* has two PsbO isoforms. In this work (Papers III and IV), using T-DNA insertion knockout mutants, we attempted to study their roles in PSII and determine their GTPase activity.

Previously, a PsbO1 lacking mutant, that has a single point mutation, was shown to have retarded growth, light green leaves and a lower PSII content (Murakami et al., 2002; 2005). Two knockout mutants of each of the psbO genes were obtained from the SALK Institute; mutant lacking PsbO1 (SALK 093396), called *psbO1* with an insertion in the 3’-UTR region, and a mutant lacking PsbO2 (SALK 024770) called *psbO2*, with an insertion in one of the exons. The *psbO1* showed similar phenotype as Murakami’s knockout mutant, while the *psbO2* had dark green leaves slightly elongated (Paper III).

Immuno detection of PSII proteins (CP43, D1, D2, Lhcb2 and PsbP) showed an overall reduction (75%) in *psbO1* and increased (125%) in *psbO2* as compared with wild type (WT), while PSI content did not significantly differ among mutants and wild type. This would explain their visual difference seen between the mutants and wild type plants. To analyse the photosynthetic performance, we measured the oxygen evolution in thylakoids from *psbO1* and *psbO2*. The obtained values, 70% and 145% respectively to WT, matched their PSII content, while activity from LHCII-PSII supercomplexes did not significantly vary. This indicates that both of the PsbO proteins can stabilise the OEC and support the oxygen evolution but in various degrees under stress light conditions. Phenotypic-response due to high light stress of plants, showed that the average leaf weight was reduced with the most severe effect on *psbO2* under extended high light, pointing to a critical role for PsbO2 protein in PSII D1 repair cycle. To confirm this, we measured PSII activity and D1 degradation in thylakoids subjected to light stress, see figure 5 in paper III. The important finding that the D1 degradation was impaired in *psbO2*, implied that PsbO2 may be essential for the initial steps of D1 degradation during high light stress. As described by (Aro et al., 2005), reversible phosphorylation of PSII proteins regulates the functional stability of PSII complexes, and that dephosphorylation of D1 protein is a prerequisite for its degradation (Rintamaki et al., 1996). Interestingly, the *in vivo* phosphorylation levels of the PSII D1 and D2 protein in the *psbO1* were significantly lower (60%) compared to that of WT and *psbO2* knockout. This was confirmed by mass spectrometry. The endogenous level of protein phosphorylation is the result of phosphorylation/dephosphorylation reactions. To investigate the mechanism behind the lower phosphorylation levels in *psbO1*, we studied *in vitro* phosphorylation
and dephosphorylation, see figure 7 and 8 from paper III. We could conclude that \textit{psbo1} had a faster D1 dephosphorylation and degradation in thylakoids as compared to wild type and \textit{psbo2}.

### 3.4.7. \textit{Arabidopsis} PsbOs differ in their GTPase activity

In paper IV, we have investigated the GTPase activity of \textit{Arabidopsis} PsbOs in the corresponding knockout mutants, \textit{psbo1} and \textit{psbo2}. As in paper II, isolated PSII membranes and NaCl washed PSII membranes were incubated with [\(\alpha\)-\textit{32P}]GTP in darkness at pH 6.0. We calculated the amount of hydrolysed GTP to GDP expressed first per mg chl and then per mol protein, see figure 1 in paper IV. The PsbO2 protein in \textit{psbo1} showed a three fold higher activity as compared to the PsbO1 protein in \textit{psbo2}.

Following GTP hydrolysis, the inactive GDP-form may be released from the membrane (figure 5, step 4-5). Previously, (Hundal et al., 1990), showed that PsbO dissociates from its docking site upon photoinactivation of the PSII electron transport. Figure 2 paper IV, show the release of \textit{Arabidopsis} PsbOs from illuminated PSII membranes under various light conditions and in the presence with or without GTP. PsbO2 protein is more efficiently released in the \textit{psbo1} compared to WT and the PsbO1 protein in \textit{psbo2}.

\textit{Arabidopsis} PsbO1 protein has a structural role in the stabilisation of the OEC complex while the PsbO2 protein regulates the PSII turnover. The reasons for their differential roles are not known. PsbO1 and PsbO2 differ in 11 amino acids, of which two amino acids are located in the targeting sequence. Previously, (Murakami et al., 2005) showed that mutation of three amino acids between PsbO1 and PsbO2 in the C-terminal region, could affect oxygen evolution. Six out of the remaining nine are in or close by the GTP-binding domain, and only one is not conserved. The change from an Ala131 (PsbO1) -> Thr (PsbO2 as well as spinach PsbO), i.e., non polar to polar amino acid change in the G1 domain, could explain the change of the GTPase activity between \textit{psbo1} and \textit{psbo2}.

Most striking is that the putative GTP-binding motifs are completely absent or only partially conserved in \textit{Chlamydomonas} (eukaryotic unicellular, green alga) and \textit{Synechococcus} (prokaryotic, cyanobacterium) PsbO, allowing us to suggest that GTP-binding to PsbO is a plant-specific feature, as is the GTP-dependent D1 protein degradation (Spetea et al., 1999; paper II)
3.5. ATP and phosphate thylakoid transporters

![Figure 9](image.png)

**Figure 9.** An updated overview of transporters related to nucleotide metabolism in chloroplasts. Modified from Spetea and Thuswaldner (2008), with kind permission from Cornelia Spetea (Linköping University).

3.5.1. Thylakoid ATP transporter

In paper I, we provide evidence of nucleotide transport in the chloroplast thylakoid membrane, by using the luminal NDPK activity, which can form GTP using externally added ATP. Immuno blotting and transport inhibition, using an antibody against a bovine ATP/ADP carrier indicated that the responsible 36.5 kDa protein in spinach is a homologous of the bovine ADP/ATP carrier.

This protein responsible for the activity described in paper I has recently been identified as the product of the At5g01500 gene and functionally characterised as a thylakoid ATP/ADP carrier (TAAC) in *Arabidopsis* (figure 5, step 1) (Thuswaldner et al., 2007).

3.5.2. Thylakoid phosphate transporter

An active nucleotide metabolism in the thylakoid lumen, reported in paper I, implies the existence of additional transporters (figure 9) than TAAC, such as those recycling inorganic phosphate (Pi) to the soluble stroma (figure 5, step 5). Among the transporters in the chloroplast envelope there are several translocators for Pi, which all functions as antiport systems using Pi or
phosphorylated C3- and C6-compounds as counter substrates (Flugge, 1999; Rausch and Bucher, 2002).

In *Arabidopsis thaliana* there are six genes encoding for anion transporters (ANTR1-6) homologous to the membrane Na⁺-dependent phosphate transporter NaPi-1 (Werner et al., 1998; Reimer and Edwards, 2004). ANTR1-3 have been predicted as chloroplast proteins (Roth et al., 2004). ANTR2 has been characterised as an envelope protein by proteomics and immunodetection (Rolland et al., 2003; Roth et al., 2004). ANTR1 has been localised, by transient expression of green fluorescent protein fusion construct, to the chloroplast (Roth et al., 2004).

In paper V we show, using two different peptide specific antibodies that the *Arabidopsis* ANTR1 is a thylakoid membrane protein. The recombinant expressed ANTR1 facilitates Na⁺-dependent Pi transport into *Escherichia coli* (*E-coli*).

Structural analyses of the 512 amino acids of the ANTR1 protein, show a high score for a putative transit peptide; the theoretical mass for its processed form is 51 kDa and shows an 80% similarity with the ANTR2 protein. NaPi-1 transporter have been predicted to have 12 trans-membrane domains (Werner et al., 1998; Reimer and Edwards, 2004). Prediction of ANTR1 topology at AREMEMNON showed 8-12 putative trans-membrane domains. See figure 1A-B in paper V. The ANTR1 protein has a 50% similarity to the rabbit NaPi-1 transporter and 60% to VGLUT2 transporters.

Recently VGLUTs have been shown to transport glutamate as well as Pi by two independent mechanisms (Juge et al., 2006). To verify if ANTR1 also transports glutamate, an uptake study of L-[3,4-³H]glutamate in *E-coli*, figure 4, 6A and table 1 in paper V, showed that glutamate can bind to but is not transported by ANTR1. To further establish a physiological role of ANTR1 in chloroplast Pi metabolism, phenotypic analyses by using knockout mutants are required.
CHAPTER 4

4. Conclusion

Paper I

- The thylakoid lumen of spinach and Arabidopsis contains NDPK3, which converts ATP and GDP to ADP and GTP.
- Spinach PsbO can bind GTP
- ATP transport occurs across the spinach thylakoid membrane into the lumen via a protein homologous to the bovine ADP/ATP carrier.

Paper II

- Four GTP-binding motifs have been identified in spinach PsbO
- MgGTP induces specific changes in the structure of the spinach PsbO protein
- Spinach PsbO has a low intrinsic GTPase activity, which is enhanced when it is associated with the PSII complex in its dimeric form

Paper III

- Both Arabidopsis PsbO proteins are able to support oxygen evolution
- PsbO1 can not substitute for PsbO2 in D1 protein turnover under high light stress

Paper IV

- Between the two PsbO proteins (PsbO1 and PsbO2) in Arabidopsis, PsbO2 is the main GTPase

Paper V

- Arabidopsis ANTR1 is a membrane transporter, located in the thylakoid membrane transporting phosphate out of the lumen into the stroma.
The findings of this work, as summarised above, bring multiple evidence for nucleotide dependent processes in the thylakoid lumen and relevant transporters in the thylakoid membrane. Against old paradigms, this work provides a new and complex view of this subcellular compartment, displaying an active nucleotide metabolism with roles in the PSII D1 turnover as well as other crucial thylakoid-associated processes, yet to be identified and characterised.
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NCBI. National Center for Biotechnology Information.


